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MARINE VIBRIOS ALSO POSSESS *ARS* OPERON: MOLECULAR CHARACTERIZATION OF FOUR ARSINIC RESISTANT VIBRIOS FROM GOA, INDIA

NEELAM SINGH*, PANKAJ VERMA AND S.K. DUBEY

[1] *Laboratory of Microbial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Taleigao plateau, Goa, India-403206,*
[2] *Molecular Biology Unit, National Centre for Cell Science, Pune University Campus, Ganeshkhind, Pune , India- 411007*

ABSTRACT

Arsenic resistance in bacteria is basically due to a detoxification system encoded by the *ars* operon. It has been reported in various grams negative as well as gram positive genera. The *E. coli* chromosomal *ars* operon and *E. coli* plasmid R773 borne *ars* operons were the first ones to be discovered. The *ars* operon *Staphylococcus aureus* plasmid pI 258 which shows only 30% structural homology with the *E.coli* counterpart is representative of gram positive *ars* operons and is known to show functional homology with the former. Subsequently, the *ars* operon genes were found in diverse taxa. E.g. *Bacillus, Pseudomonas, Acidiphilium multivorum, Corynebacterium, Streptomyces, Thiobacillus, Exiguobacterium, Clostidium, Synechocystis* and many others. But this inherent property of arsenic resistance and the presence of *ars* operon have not yet been reported among the members of the genus *Vibrio*. We, in our studies came across four Vibrios isolated from marine habitats which showed high levels of resistance towards inorganic arsenic species viz. arsenate (>200mM) and arsenite(3mM). All these strains were PCR positive for *ars*C gene encoding arsenate reductase using gene specific primers. The size of the amplicon ranged from 353-356 bp and revealed 98-99% homology with the chromosomal *ars* operon *E.coli* strain W 3110.

KEY WORDS : arsenate, arsenite, efflux, amplicon, marine vibrios, *ars* operon, arsenic resistance

NEELAM SINGH
Laboratory of Microbial Genetics and Environmental Biotechnology, Department of Microbiology, Goa University, Taleigao plateau, Goa, India-403206,

INTRODUCTION

Arsenic resistance is a common phenomenon among bacterial domain. The property was known since 1960's, but the correlation between arsenic reduction and the genetic determinants was discovered only in 1982 [1]. The arsenic resistance system in bacteria is made up of three to six genes of which some can be present in multiple copies or may lie outside the operon. Generally the *ars* operon consists of five genes, *ars R*, *ars D, ars A, ars B and ars C* (*ars RDABC*) [2,3,4]. The *ars R* and ars *D* genes encode two different regulatory proteins whereas ars *A* and ars *B* genes encode subunits of an ATP driven transmembrane arsente efflux pump [5]. The last gene of the Operon, ars C is an important representative of diverse ars operons which encodes arsenate reductase. Arsenate reductase carries out the first step in arsenate metabolism in microbes i.e. reduction of As (V) to As (III) before it's efflux from the cell [6, 7.This] gene is of great importance since its product is a soluble cytoplasmic enzyme which makes possible the elimination of intracellular arsenate via reduction. Arsenate is the thermodynamically favourable form of arsenic under aerobic conditions [8, 9], so it is likely to be the most common form in many environments. Sun et al.[10] have used the PCR based screening of *ars C* gene among the arsenic resistant bacteria from environmental samples. A clear understanding of genetic systems of microbes for biotransformation of arsenic is of immense importance for designing bioremediation devices and also for biomonitoring by means of biosensors. The presence of *ars* operon has been reported in diverse genera e.g. *Bacillus, Pseudomonas, Acidiphilium multivorum, Corynebacterium, Streptomyces, Thiobacillus, Exiguobacterium, Clostridium, Synechocystis, Micrococus, Metananobacterium and Shewanella sp.* But there is no report of arsenate reduction or the presence of *ars* operon in the members of genus Vibrio. In the present communication we report the isolation of four different species of *Vibrio* from the west coast Goa and also confirmed presence of *ars*C gene in these isolates.These bacterial isolates were identified upto species level by 16s rDNA sequencing and NCBI – BLAST search[11].

MATERIALS AND METHODS

(i) Water sample collection and isolation of arsenic reducing microorganisms

Water samples were collected from about 0.1-1 metre depth in sterile BOD bottles using thoroughly cleaned buckets from sewage (St. Inez Nallah), Goan beaches viz. Dona Paula, and Majorda and Mandovi estuary (Table 1). Arsenic resistant vibrios were isolated on Mineral salts agar (with 3% NaCl) containing 10mM sodium arsenate and were subsequently maintained on TCBS agar (with 3% NaCl).

(ii) Identification of the arsenic resistant strains

Four potentially arsenic tolerant isolates were subsequently identified by both biochemical and molecular (16s rRNA) methods.

Morphological, physiological and biochemical characterization

In order to identify the arsenic tolerant isolates they were initially characterized with reference to their gram reaction, colony morphology and other characteristics.Cell form and cell size were determined by growing the isolates on a basal medium containing 1.0% glucose, 1.0% glycerol, 1.0% ethanol, 1.0% peptone, and 0.5% yeast extract. If necessary, 1.5% agar was added in the preparation of the solid media. Gram staining was carried out by the method of Hucker and Conn[12]. Motility was observed by the hanging-drop method, and flagellation was examined by the staining method of Toda[13]. The activity of catalase and oxidase was determined by the methods described by Navarro and Komagata[14, 15]. All other biochemical tests were performed according to the guidelines given in the Bergey's Manual of Systematic Bacteriology Vol II [16].

Molecular identification of isolates (16S rDNA sequence analysis)

For PCR amplification of the 16S rRNA gene, a small speck of the bacterial colony was resuspended in 100 µl of sterile deionised water(SDW), mixed and lysed at 90°C for 10 min. Crude lysate (0.5 µl) was added to 19.5 µl SDW and used as a PCR template. Universal bacterial 16S rRNA gene primers 27 forward and 1525 reverse [17] were used to amplify the ~1.5 kb 16S rRNA gene fragment. Following components were added to each PCR template, 10pM of each primer, 0.2mM of each deoxynucleoside triphosphate and 2.5 U of *Taq* DNA polymerase (Bangalore Genei, Bangalore, India) with reaction buffer(10X) supplied by the manufacturer. The reaction volumes were made up to 100 µl with SDW. Lysed *Escherichia coli* cells and 20 µl of SDW were used as positive and negative controls, respectively. There was an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1min and 72 °C for 1 min with a final extension at 72 °C for 10 min. An aliquot (10 µl) of the PCR product was mixed with 2 µl DNA loading buffer and analysed by electrophoresis (15 V/cm; 60 min) on 0.7% horizontal agarose gels (containing 0.5 µg/ml ethidium bromide) in Tris-borate-EDTA buffer and visualised on an UV transilluminator. The identities of the amplicons were confirmed by comparison of the amplicon sizes with the 1 kb plus DNA ladder (Invitrogen). PCR products were prepared for sequencing using a QIAquick PCR purification kit (Qiagen, USA). About 100ng of purified PCR product was sequenced directly by using an ABI PRISM™ Big Dye terminator v3.1 Cycle Sequencing Kit and the run was carried out in Applied Biosystems 3730 DNA Analyzer using internal overlapping primers [18].

Phylogenetic analysis

Contiguous 16S rDNA sequences were assembled using Chromas Pro v 1. 33. These sequences were initially analyzed at NCBI server RDP databases using BLAST [11] tool and similarity rank (Maidak et al.) algorithms and corresponding sequences were downloaded. The multiple alignments were performed by the program ClustalX v1.81[20] and manual corrections were made in DAMBE v4.5.33 [21]. The phylogenetic and molecular evolutionary analyses were conducted using MEGA v3.1[22] in neighbor joining algorithm[23] and Kimura 2 parameter. The comparison of the DNA sequence data obtained was made on 1,381 bases. The robustness for individual branches were estimated by bootstrapping with 10,000 replicates[24] to get a strict consensus tree, using *Photobacterium angustum* (X74685) as an outgroup. Evolutionary genetic distances of four bacterial strains were calculated using pairwise sequence alignment program of MEGA v3.1 with Kimura 2 parameter [25] and taking into account 1,305 sites (Fig.1 , Table 2).

RESULTS

1. Arsenate tolerance limits

The tolerance limits were determined in MSM + 02% glucose supplemented with 1.5% NaCl and increasing concentrations of sodium arsenate. All the four *Vibrio* strains SI 9, Maj 4, Man 2 and BL9 could grow on plate containing 250 mM of sodium arsenate. The 16s ribosomal RNA gene sequences obtained in this study have been deposited in the GenBank database under accession no. DQ513192, DQ980029, DQ513193 and DQ985231 (Table 2).

Table 1
Geographical location of the sampling sites

S.No	Sampling site (Source)	Geographical location	Bacterial strain
1	Saint Inez Nullah	N 15º 29' 54.1" E 073º 49' 12. 3"	*Vibrio* sp. SI9
2	Majorda Beach	N 15º 18' 39.8" E 073º 54' 05.4"	*Vibrio* sp. Maj4
3	Dona Paula	N15°27' 04.06" E 073° 48' 11.09"	*Vibrio Campbellii* BL9
4	Mandovi Estuary	N15° 30' 05.06" E 73° 49' 42.6''	*Vibrio aesturianus* Man2

Table 2
Molecular identification of the arsenate resistant strains by 16s rDNA

S.NO.	Bacterial Strain	Identification	GenBank Accession no.
1	SI9	*Vibrio* sp SI9(similar to *Vibrio* sp.NAP4)	DQ 513193
2	BL9	*Vibrio campbellii*	DQ 980029
3	Maj4	*Vibrio* sp SI9(similar to *Vibrio* sp.CJ 11052)	DQ 513192
4	Man2	*Vibrio* sp.(similar to *Vibrio* sp. BWDY-52)	DQ 985231

Table 3
GenBank Accession Numbers of the arsenate reductase sequences of Vibrio strains

S.No.	Name of the Strain	GenBank Accession No.
1	*Vibrio* sp. strain SI9	EF375944.1
2	*Vibrio campbellii* strain BL9	EF375942.1
3	*Vibrio* sp. strain Maj4	EF375943.1
4	*Vibrio aesturianus* strain Man2	EF375945.1

1. PCR amplification of arsC gene

The *ars*C gene of the *ars* operon was amplified using the primers *amlt*42 F and *amlt*376 R[26] using the plasmid as well as chromosomal DNA of the four isolates. The sequence of the 353-356 bp amplicon was determined by using automated sequencer (ABI PRISM sequencer). All the *ars*C sequences were also deposited to the GenBank and accession numbers acquired (Table 3). The PCR primers specific for *ars*C gene are:

Amlt42 F 5'-TCGCGTAATACGCTGGAGAT-3'
Amlt376 R 5'-ACTTTCTCGCCGTCTTCCTT-3'

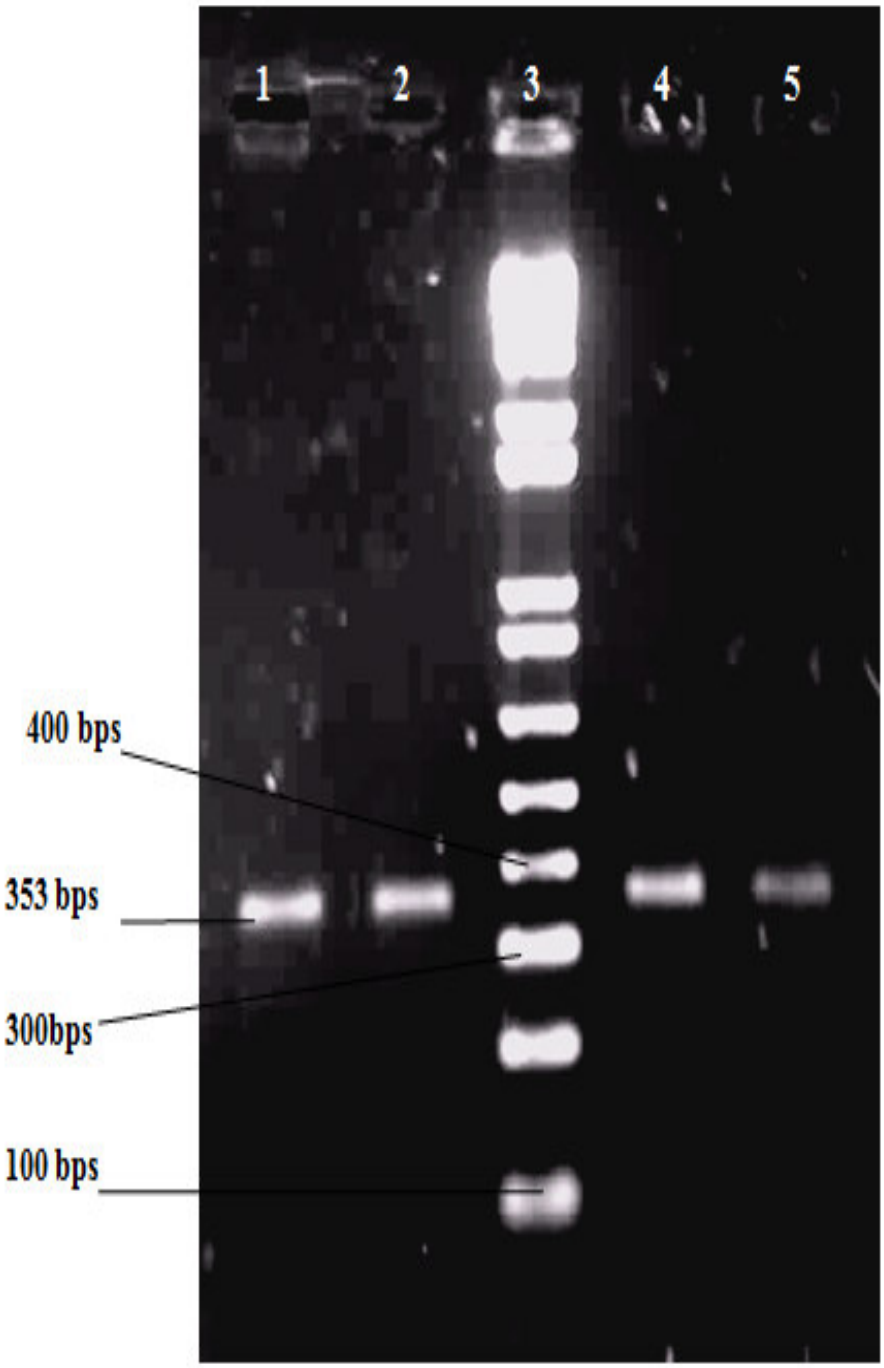

Figure 1
PCR amplification of arsC gene (353-356 bp) in four arsenate tolerant strains

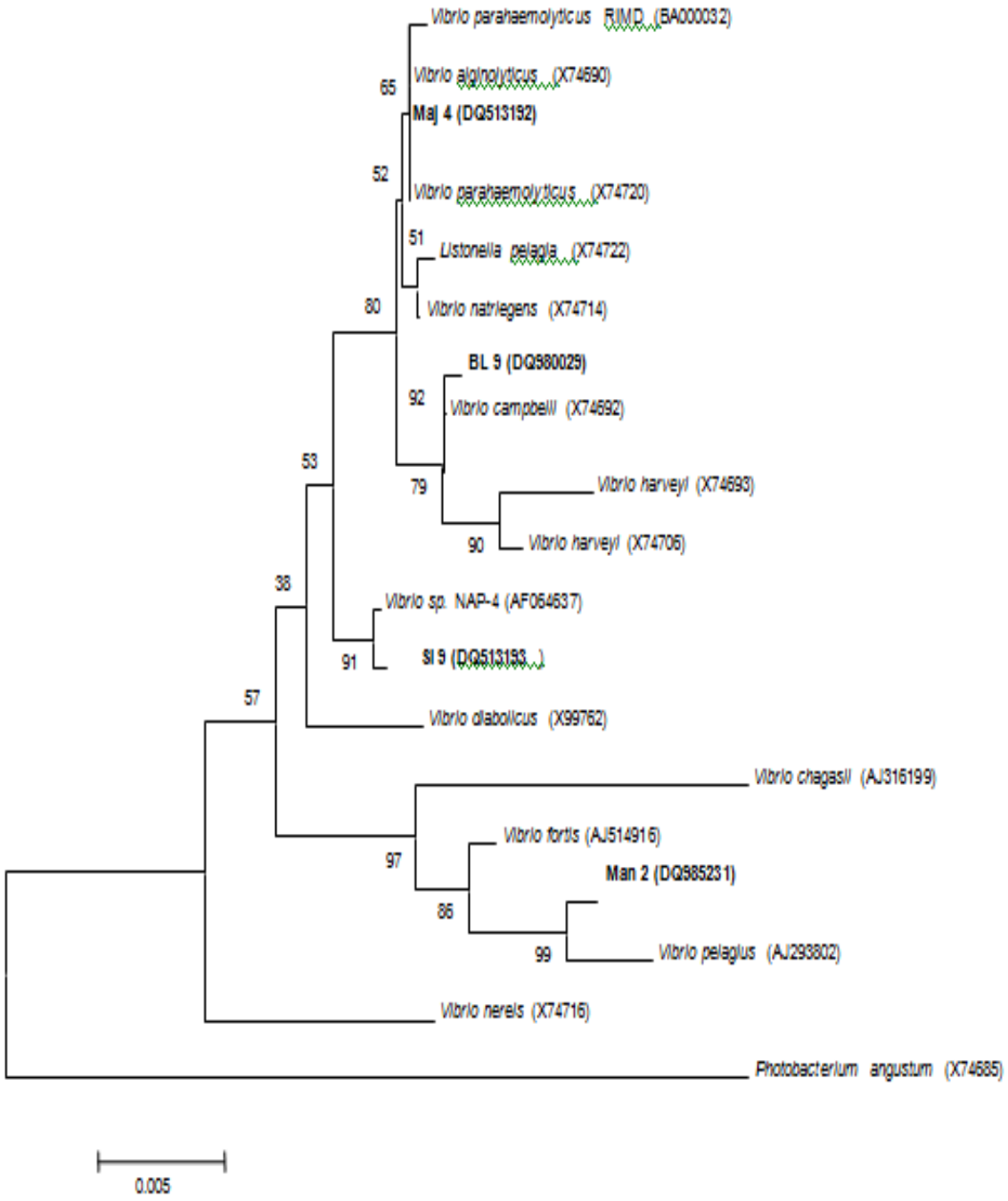

Figure. 2

Phylogenetic tree based on 16S rRNA gene sequence comparisons over 1,381 bases showing the relationship between members of genus Vibrio and isolates Maj4, BI9, SI9 and Man2. *Photobacterium angustum* has been taken as out-group. The sequences have been retrieved from NCBI database and the tree has drawn using neighbor-joining method in MEGA software. The scale bar represents 5 nucleotide substitutions per 1000 nucleotides and values at nod represent percentage of 10,000 bootstrap replicates. Numbers in bracket represents GenBank accession numbers.

DISCUSSION

The *ars* operon was reported for the first time in *Staphylococcus aureus* plasmids pI 258 and pSX267[27, 28, 6]. The *E.coli* plasmid borne *ars* operon was sequenced by Chen et al.(1986)although the presence of resistance was known since 1970's. The arsenic resistant plasmid R773 was discovered in 1970 and was studied in detail by Silver et al.[29] in terms of inducibility of arsenic resistance by various metal ions such as arsenate, arsenite, antimonite, Selenite and Bismuthite etc. The complete sequence of the plasmid borne[30] and chromosomal *ars* operon was determined in the subsequent years[31,32,33]. A chromosomal *ars* operon was discovered in *Pseudomonas aeruginosa* in the following years [34]. In the subsequent years this operon has been discovered in a wide range of diverse genera among the entire bacterial kingdom e.g. *Acidiphilium multivorum*[35] *Bacillus subtilis*[36] and *Thiobacillus ferrooxidans* [37] and *Aeromonas punctata* [38]. The *ars* genes may either be located on plasmid or on the chromosomal genome or on both [39]. The *ars* operons of gram negative share highly homologous sequences. Sequences homologous to *E.coli ars* operon are also highly conserved among enterobacterial genera[33]. Molecular characterization (16s rDNA sequencing and NCBI-BLAST) of all four vibrio isolates revealed that isolate SI9(DQ 513193) resembles *Vibrio* sp. NAP-4 (AF064637.1), BL9(DQ980029) with *VIbrio campbellii* (X74692.1), Maj4(DQ513192) with *Vibrio* sp. CJ11052 (AF500207.1) and Man2 with *Vibrio* sp. BWDY 52 (DQ328953.1) respectively (Table 2).it is important to notice that though all the four arsenic tolerant isolates are distantly apart phylogenitically but they all posssess *ars*C gene in their *ars* operon (Fig.1, Fig.2).It is interesting to report that the *ars*C sequences of all these four marine Vibrios are much homologous to chromosomal *ars*C sequences of *E.coli* strains, showing 99% homology with the chromosomal *ars*C gene of *E.coli* W3110 and *E.coli* K12 which indicates that both have a common phylogenic origin. All of our vibrio isolates positively amplified *ars*C gene amplicon (353-356 bps) when gene specific primers for *E.coli ars*C were used (Fig. 2) and upon sequencing confirmed the presence of *ars*C gene (Table 3).

CONCLUSION

The biochemical and molecular studies of these four arsenic tolerant vibrio isolates clearly revealed that in spite of being phylogenitically distant from each other they all possess *ars* operon (*ars*C gene) which is interestingly 99% homologous to chromosomal *ars*C gene of *E.coli* W3110.The inducible promoters of these marine vibrios can possibly be used to construct biosensors and be used for environmental biomonitoring.

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ABBREVIATIONS

TCBS= triple sugar bile salt sucrose
SDW= sterile distilled water
MSM= mineral salts medium

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